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Microbial decomposition of pentachlorophenol

EDWIN J. KIRSCH AND J. E. ETZEL

PENTACHLOROPHENOL (PCP) is a biocide widely used in processing wood and other cellulosic products, starches, adhesives, proteins, leather, oils, paints, and rubber. It has been used to control undesirable fungi, bacteria, insects, weeds, mollusks, and other nuisance biota.[1] In current practice, pentachlorophenol is primarily used as a wood preservative. Although under certain conditions it may be considered an environmental pollutant having potentially hazardous properties commonly ascribed to chlorinated hydrocarbons, it is not disseminated broadly over fields and crops as are the common agricultural pesticides. Pentachlorophenol is localized in treated wood and owes its lasting preservative effect to its resistance to biological or chemical degradation and leaching. Nevertheless, potential pollution arises mainly during its manufacture and in the wood treatment plant where the environment may become polluted during processing. It is therefore of interest to investigate the potential for biodegradation of pentachlorophenol by soil or aquatic microorganisms.

Information regarding the biodecomposition of PCP is relatively scarce and quite vague. Toxicity and biocidal activity of PCP have been shown to disappear in soil. The extent of disappearance depends on the type of soil, moisture content, temperature, and sunlight.[2-5] Kincannon *et al.*[6] were unable to observe disappearance of PCP in activated sludge systems although PCP-acclimated sludge was considerably more tolerant to PCP than unacclimated sludge. Ingols *et al.*[7] attempted to acclimate activated sludge to PCP but were unable to demonstrate ring degradation or chlorine removal. Suggestive information related to PCP biodegradation by three species of *Trichoderma* has been reported by Cserjesi,[8] who measured disappearance of 88 percent PCP over a 12-day incubation period in malt extract solution.

The purpose of this investigation was to obtain definitive evidence of PCP biodegradation in heterogeneous cultures of microorganisms.

MATERIALS AND METHODS

Source of culture. A soil sample was obtained from the grounds of a wood products manufacturer in Terre Haute, Ind., who used pentachlorophenol as a preservative. The sample was obtained near the fence-post drying racks where the soil had been well saturated with chemical wood preservatives. A 50-g portion of soil was added to 4 l of 0.1 percent nutrient broth in an 8-l cylinder. The culture was stirred mechanically and aerated by passing compressed air through a sintered glass sparger. Abundant growth was observed after 24 hr incubation at room temperature.

Acclimation of culture to pentachlorophenol. An acclimation regime was initiated that involved daily removal of 800 ml of settled culture liquor and the replacement of this volume with fresh nutrient broth, 1 g/l, containing 80 mg phenol and 10 mg pentachlorophenol. The liquid retention time was 5 days. At each 5-day interval the concentration of phenol was increased until after approximately 1 month the daily increment of phenol was 1.28 g/day. At this point the incremental addition of phenol was decreased and the increment of pentachlorophenol was increased so that by the third month of acclimation the daily input of phenol was reduced to zero and pentachlorophenol was being added at 120 mg/day. The level of nutrient broth added

remained unchanged throughout the acclimation period. The acclimation culture was maintained with nutrient broth and pentachlorophenol for an additional 4 months before radioisotopic studies was begun.

Preparation of radioactive pentachlorophenol. Pentachlorophenol-U-^{14}C was prepared by exhaustive chlorination of phenol-U-^{14}C and purified by fractional sublimation as described by Rogers *et al.*[9] Early biodegradation experiments were done with pentachlorophenol that was shown to be 97.0 percent radiochemically pure. This material melted at 187.1°C, as compared with 191°C for an authentic pure compound. Later in the study, after a second sublimation succeeded in removing a small radiochemical impurity, the pentachlorophenol used was found to be 99.55 percent pure. The purest labeled compound obtained melted at 190.4°C and had a specific activity of 0.270 μci/mg.

Radioactive assays for biodegradation of pentachlorophenol. Mixed bacterial cell preparations were obtained for assay of pentachlorophenol oxidation capacity directly from the acclimation culture or from shaker flask cultures inoculated with the enriched culture. In the latter situation, the growth medium contained NH_4Cl, 2.0 g; Na_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; yeast extract, 0.05 g; phenol, 0.1 g; pentachlorophenol, 0.01 g; deionized water, 800 ml; and tap water, 200 ml; in 50-ml Erlenmeyer flasks. The medium was adjusted to pH 7.8 before sterilization. Before an assay was started, the cells were centrifuged at 4°C, washed once in 0.02 *M* phosphate buffer at pH 7.8, and suspended in buffer. Unless otherwise noted, the optical density of each cell suspension was adjusted to a nominal value of 1.5 using a spectrophotometer at 600 nm. It was determined that an optical density of 1.5 was equivalent to approximately 1.5 mg dry cells/ml. Except where noted in the text, all assays were done at equivalent cell densities.

Warburg flasks (15-ml volume) were used as reaction vessels in all radioisotope experiments. The temperature of the water bath was maintained at 25°C, and shaking rate was 120 strokes/min. The flasks were charged with 3.0 to 4.0 ml of cell suspension and 0.25 to 1.0 ml pentachlorophenol-^{14}C as the sodium salt containing 35,000 to 45,000 dpm and representing 37.5 to 75.0 μg of organic substrate. An 8- by 25-mm strip of filter paper saturated with hyamine hydroxide was placed in the center well to trap the radioactive CO_2 evolved.

Ultimate biodegradation of pentachlorophenol was determined as the amount of radioactive CO_2 evolved relative to time. Pentachlorophenol oxidation capacity of the cells was defined to mean the amount of radioactive CO_2 released in 24 hr as a percent of the total substrate radioactivity in the assay flask.

The radioactive carbon dioxide trapped in hyamine hydroxide as hyamine carbonate was measured by placing the paper strip directly into a scintillation vial containing 15.0 ml of 0.4 percent 2,5-diphenyloxazol in a toluene:ethoxyethanol, 1:1 solvent. Ten-minute radioactive counts were made with a scintillation counter equipped with a bialkaline phototube. Counts are corrected for background and counting efficiency and are reported in absolute units as disintegrations per minute.

RESULTS

Table I presents data indicating that an acclimated mixed microbial population is capable of attacking pentachlorophenol under nonproliferating conditions. That biological attack results in terminal oxidation of this halogenated phenol is indicated by the significant release of radioactive carbon dioxide in a 24-hr incubation period. These data tend to be conservative estimates of the potential ability of acclimated bacteria to decompose pentachlorophenol, because they are taken from early experiments. More recent work, which will subsequently be described, has shown that as much as 68 percent of the radioactive pentachlorophenol carbon added can be recovered as radioactive CO_2 in 24 hr. It is important to note that insignificant radio-

active CO_2 was released spontaneously under the conditions of this experiment. These and other data obtained confirm that pentachlorophenol may undergo biologically mediated decomposition.

Various nutritional components used in the growth medium for developing a PCP-acclimated mixed culture were studied for their effect on the initial rate of PCP oxidation and to determine whether PCP oxidation was influenced by conditions that might support cell proliferation. Cells were taken from an acclimated culture, centrifuged, washed, and placed in Warburg flasks with radioactive PCP. The cell dry weight/flask was 10.4 mg in a total reaction volume of 5.0 ml. Phenol at 20 mg/l and nutrient broth at 1,000 mg/l were added separately and in combination. For comparative purposes, a control oxidation system containing washed cells, PCP-^{14}C, and no additives was established. The reaction vessels were incubated for a total of 46 hr, and $^{14}CO_2$ evolved was determined at intervals through the incubation period. The data obtained are presented in Table II. Several important observations may be made. The overall recovery of PCP carbon as CO_2 was approximately 50 percent for resting cells; this value was obtained for either cells with PCP alone or cells with PCP and phenol. Phenol, at the concentration used, apparently had little or no effect on the rate and extent of oxidation of PCP. However, when organic nitrogen was available as supplied by nutrient broth and the cells were in a dynamic growth condition, the overall oxidation of PCP seemed retarded by a factor of two. In addition, the interval rates of oxidation were quite consistently lower, particularly during the later stages of incubation. There was some reduction in immediate oxidation rate as well. Clearly, PCP was oxidized by growing and resting cells, but the data suggest that favorable growth conditions are not necessarily favorable to PCP oxidation when mixed cultures are used.

TABLE I.—Biological Decomposition of Pentachlorophenol to Carbon Dioxide

Reactants	Reaction Time (hr)	$^{14}CO_2$ Collected (dpm/24 hr)	Pentachlorophenol (Recovered as CO_2) (%)
Pentachlorophenol-^{14}C plus washed cells	24	9,207	14.0
Pentachlorophenol-^{14}C plus boiled washed cells	48	19	0.06
Pentachlorophenol-^{14}C with no cells	90	29	0.05

TABLE II.—Effect of Culture Medium Components on Rate of Pentachlorophenol Oxidation

Substrates	Accumulated Time (hr)	$^{14}CO_2$ Evolved Cumulative Count (dpm)	Recovery $^{14}CO_2$ (%)	Interval Rate $^{14}CO_2$ Evolution (dpm/hr)
PCP-^{14}C—30 mg/l	3	1,852	2.9	617
	7	4,959	7.7	750
	12	9,379	14.5	900
	20	17,349	27.0	1,000
	46	32,288	50.0	575
PCP-^{14}C—30 mg/l + nutrient broth —1,000 mg/l	3	1,452	2.2	485
	7	3,353	5.2	475
	12	5,881	9.1	505
	20	9,150	14.3	410
	46	13,981	21.6	185
PCP-^{14}C—30 mg/l + phenol—20 mg/l	3	1,727	2.7	575
	7	4,544	7.1	700
	12	8,390	13.0	770
	20	15,862	24.6	934
	46	29,701	46.0	534
PCP-^{14}C—30 mg/l + nutrient broth —1,000 mg/l + phenol—20 mg/l	3	1,192	1.8	398
	7	2,690	4.2	374
	12	4,254	6.6	312
	20	7,737	11.9	436
	46	13,602	21.0	226

The effect of cell concentration on the rate of PCP oxidation by resting cells is described in Table III. Cells were harvested and concentrated from a PCP acclimation culture that was oxidizing PCP at a rate of 10 to 15 percent of added substrate in 24 hr. Dilutions of the cell suspension were exposed to PCP-^{14}C. Carbon dioxide liberation was measured at frequent intervals through the incubation period. An average rate of PCP oxidation was obtained and used to calculate the oxidation rate per milligram of cells (specific oxidation rate). The initial activity of these

TABLE III.—Effect of Cell Concentration on Pentachlorophenol Oxidation

Cell Concentration (mg dry wgt/flask)	Average Rate of $^{14}CO_2$ Release (dpm/hr)	Specific Rate of $^{14}CO_2$ Release (dpm/hr/mg cells)
1.5	53	35
4.6	166	36
13.9	350	25

cells was considerably lower than that of the ones used in the previous experiment. Consequently, the absolute rates of PCP decomposition are not consistent. The experiment was intended to show the proportionality between cell weight and rate of PCP oxidation. The data constitute important ancillary evidence of the biological involvement in PCP oxidation. The specific rate of PCP oxidation was 35 and 36 dpm/hr/mg cells at the lowest and intermediate level of cells. However, at the highest cell level the oxidation rate increased only twofold for a threefold increase in cells. While this increase is in the right direction, it suggests some limitation in oxidation. Probably this limitation involves oxygen deprivation, because the cell suspension at 13.9 mg/flask was visibly heavy, and the authors have shown in experiments not reported here that a decreased shaking rate decreases the rate of PCP oxidation.

During the entire course of this research, PCP acclimation cultures were constantly maintained by supplying a small increment of nutrient broth and unlabeled PCP on a daily basis in an aerated, agitated culture vessel. This culture was handled as a fill-and-draw system rather than a continuous-flow system, and transitory variations in the PCP oxidation capacity of the biomass were observed. The term "PCP oxidation capacity refers to the percent PCP carbon evolved as $^{14}CO_2$ after 24 hr of incubation with washed cells adjusted to a uniform turbidity that was equivalent to approximately 5 mg/reaction flask. A single acclimation culture was studied over a 57-day period from initiation to failure. Daily samples of the acclimating biomass were removed and washed. The ability of the cells to oxidize PCP was determined, and selected interval data are presented in Table IV. The culture continually improved in its ability to oxidize PCP between Day 1 and Day 25. Between Days 25 and 42 a stable plateau was obtained; however, a rapid degeneration of the PCP oxidation capacity was observed between Days 42 and 47. The system was nearly completely devoid of ability to oxidize PCP by the 57th day. The exact reasons for this failure have not been established experimentally. Pentachlorophenol itself or some toxic metabolite may have accumulated in sufficient quantity to retard the PCP-oxidizing microbial population. In any case, degradation rates of sufficient magnitude to oxidize nearly 70 percent of the PCP carbon in 24 hr were maintained for nearly 2 wk during the period of peak performance.

TABLE IV.—Variations in Pentachlorophenol Oxidation Capacity of a Heterogeneous Acclimating Biomass

Sampling Time* (days)	PCP Carbon Released as CO_2 (dpm/24 hr)	Oxidative Capacity† (%)
1	4,400	10
5	5,100	11
7	9,800	22
12	20,000	44
20	17,000	38
25	31,000	68
32	31,000	68
39	29,000	65
42	27,000	61
47	17,000	38
53	4,000	9
57	50	1

* During this acclimation cycle a 4-l completely mixed, aerated reactor received 40 mg/day PCP and 500 mg/day nutrient broth.

† Percent radioactive PCP carbon converted to CO_2 in 24 hr.

DISCUSSION

The data presented support the contention that pentachlorophenol, a commercial biocide, is amenable to biological decomposition under specific idealized laboratory conditions. Radioisotopic analysis of the CO_2 released during oxidative attack by resting and growing cells indicates quite clearly that substantial decomposition of the substrate biocide is possible. The rate of oxidative attack may depend on several factors. Within a ninefold range of cellular concentration, this rate was shown to be dependent to a large degree on the cell concentration. Some rate limitation appears at the highest cell concentration used. The data presented cannot distinguish whether at high cell levels the limit-

ing factor is related to oxygen, substrate deficiency, or some other unknown factor. Certainly, the absolute rate of oxidation reported in these experiments and throughout the investigation was governed, in addition, by constancy or inconstancy of the viable fraction of bacteria in the mixed culture population capable of attacking PCP. The fraction of bacteria able to consume PCP in a given population seems to be related to the prior culture history of the mixed bacterial population. This was clearly observed in the acclimation culture, where during a 60-day incubation period a gradual evolution toward high-rate PCP oxidation was followed by a rapid decline in the PCP oxidative capacity of the culture. The data might be interpreted to mean that the PCP-oxidizing organisms, although slower growing than other organisms in the culture, gradually become a significant fraction of the population as the result of some selective culture advantage that may have been related to the gradually increasing concentration of exogenous PCP. The reason for the sharp decrease in PCP-oxidizing capacity is not known. Finally, another factor that may influence the apparent rate of PCP oxidation is the condition of cells when the analysis is made. In the resting cell suspension exposed only to PCP, oxidation of PCP is more rapid than in cells supplied organic nutrient such as nutrient broth plus PCP. This suggests that PCP is probably not a primary substrate but serves rather as a secondary substrate that does not compete favorably with more easily degraded materials. In fact, it would not be at all surprising to find that PCP is co-metabolized by actively growing cells which use some other aromatic moiety as a primary energy source.

CONCLUSIONS

1. Acclimated, proliferating, and nonproliferating mixed culture bacterial populations are capable of biodegrading sodium pentachlorophenate. Biodegradation was measured by the substantial release of radioactive carbon during a 24-hr exposure period.

2. The rate and extent of carbon dioxide liberation is highest in nonproliferating cultures where sodium pentachlorophenate is the sole carbon source.

3. The rate of carbon dioxide liberation in a nonproliferating population is proportional to the biomass concentration at low cellular levels; however, at a high cell concentration rate limitation is observed.

4. The sodium pentachlorophenate oxidation capacity (carbon dioxide liberated/24 hr/unit cell mass) of a mixed population growing in a fill-and-draw, completely mixed aerator with daily increments of dilute nutrient broth and sodium pentachlorophenate reaches a maximum of 68 percent in 25 days. The oxidative capacity remains stable for approximately 17 days and then begins to diminish to a negligible level during the next 14 days.

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Authors. Edwin J. Kirsch and J. E. Etzel are associated with the Department of Environmental Engineering, School of Civil Engineering, Purdue University, West Lafayette, Ind.

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